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Nano-bio hybrid materials for a new generation of high-throughput diagnostic systems

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Abstract

Nano-bio hybrid materials obtained by conjugation of capture molecules and plasmonic (metal) or excitonic (semiconductor) nanocrystals or microspheres encoded with fluorescent semiconductor nanocrystals of different colors are the basis for development of a new generation of high-throughput diagnostic systems. Here, the general principles of development of "ideal" diagnostic nanoprobes based on oriented conjugates of capture molecules with the nanoparticles of different chemical compositions or with optically encoded microspheres are summarized and the basic requirements for individual components of the photonic nanoprobes being developed are discussed in the context of ensuring their advantages over the existing photonic diagnostic systems.

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1. Introduction

The photonic detection employing conventional methods of labeling with organic dyes used in immunocytochemical and flow cytometry diagnostics strongly suffers from weak resistance of dyes to photobleaching, their red-tailed fluorescence emission spectra, short lifetime, and problematic interpretation of the results due to the spectral overlaps in the case of multiplexed detection with several light-emitting probes (Gao et al. (2004)). Compared with traditional organic dyes, a new generation of fluorophores, inorganic fluorescent nanoparticles called quantum dots (QDs) possess excellent and unique optical and physico-chemical properties (Samokhvalov et al. (2013); Sukhanova et al. (2004); Sukhanova et al. (2002); Bilan et al. (2015)).

The monodispersed nanosized semiconductor particles are much brighter fluorescent tags than organic fluorophores, are detectable as single nanocrystals with a routine epifluorescent microscope setup, and provide a great asset for efficient detection of low-abundance targets (Sukhanova et al. (2004); Sukhanova et al. (2002)). They can be rendered biocompatible and represent a powerful tool for direct readout of information down to the single-molecule level. Moreover, they are several thousand times more stable against photobleaching than organic dye molecules and, hence, are ideal probes for fluorescent spectroscopy and bioimaging applications (Mahmoud et al. (2011)). Despite breakthrough advances in the preparation of QDs and their applications to multiplexed optical encoding (Montenegro et al, 2013), very few applications of these tools to clinical cancer diagnostics have been published to date. Appearance of

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the recently proposed B4 principle for preparation of ultrasmall diagnostic nanophotonic probes (Fig. 1) may increase the range of their applications (Mahmoud et al. (2011); Nabiev et al. (2007); Sukhanova et al. (2012); Hafian et al. (2014)).

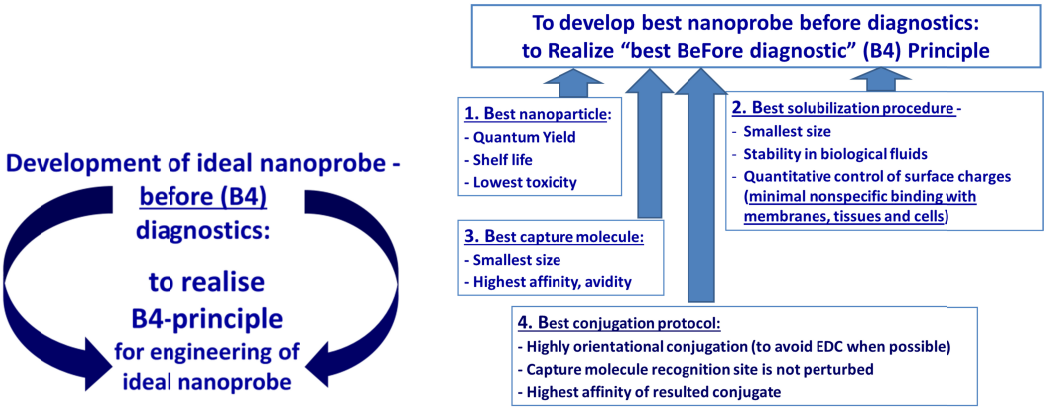

Fig. 1. The B4 (Best BeFore diagnosis) approach to the development of nanophotonic diagnostic probes, which includes the selection of (1) the best nanoparticles as visualization labels, (2) the best solubilization procedure for nanoparticle transfer to the aqueous phase, (3) the best capture/recognition molecules and (4) the best conjugation protocol for tagging recognition molecules and detecting nanoparticles together.

2. Experimental part

When QDs are used for molecular or cellular targeting, three main procedures are employed to couple them with the DNA oligonucleotide or aptamer, antibodies (Abs), or other capture molecules. In the first procedure, efficient bioconjugation may be achieved by cross-linking the QDs' amine with the biomolecule's carboxylic acid or sulfhydryl groups (Bilan et al. (2015)). The first bioconjugation method ensures highly stable covalent linkage of QDs to biomolecules and is the most commonly used approach to making biofunctionalized QDs for in vitro cell labeling and in vivo imaging. In contrast, since sulfhydryl groups are known to be unstable in the presence of oxygen, chemical modifications are often required before QD conjugation via these groups. Nevertheless, due to the specific localization of -SH groups, sulfhydryl covalent linkage provides fixed outward orientation of the target-specific Ab fragments.

In the second procedure, electrostatic interaction between QDs and charged adapter molecules or proteins with incorporated charged domains is employed. In one application of this technique of QD coupling with Abs, an adaptor protein with a positively charged leucine zipper domain is used for electrostatic binding to the QD, and a protein G domain, for binding to the Ab Fc fragment. In the electrostatic interaction approach, the binding energies considerably depend on both the chemical environment and the ambient temperature, and the QD size can strongly affect the interaction efficiency. Therefore, the conjugates made in this way are unsuitable for in vivo or ex vivo cell labeling due to the possible interference with positively charged proteins.

As a third procedure, the strong streptavidin–biotin interaction is used to ensure QD–streptavidin coupling with biotinylated proteins, notably biotinylated Abs. It should be noted that, in this case, the orientation of the bound capture molecule cannot be easily controlled and may lead, e.g., to production of QD-labelled Abs with the target-specific F(ab')2 domains facing inwards. Mahmoud et al. (2011) have published an optimized procedure for preparation of oriented conjugates of full-size Abs and nanocrystals of different chemical compositions ensuring a high concentration of capture molecules with intact recognition sites on the surface of nanoparticles after their conjugation (Fig. 2).

An alternative approach includes the use of genetically modified single-domain antibodies (sdAbs), which can be tagged with nanoparticles, via an additional amino acid residue integrated in their C-terminus, in a highly oriented manner, with all binding sites oriented outward of the nanoparticle surface (Sukhanova et al. (2012); Hafian et al. (2014)). Such nanoprobes with hydrodynamic diameter below 12 nm contain four homogeneously oriented copies of sdAbs on the surface of each QD. They exhibit excellent specificity and sensitivity in the quantitative detection of rare biomarker-expressing cells using flow cytometry. The higher diffusibility of sdAbs enables immunohistochemical analysis of thick tissues not accessible to monoclonal Abs and lead to tissue biopsy labeling of the same or even better quality than that obtained with the gold standard immunohistochemical diagnostics. Additionally, the conjugates are bright multiphoton nanoprobes enabling clear discrimination of CEA-overexpressing tumor areas from normal tissue. The sdAb–QD nanoprobes developed have numerous implications for high-throughput multiplexed diagnostics and FRET-based detection platforms.

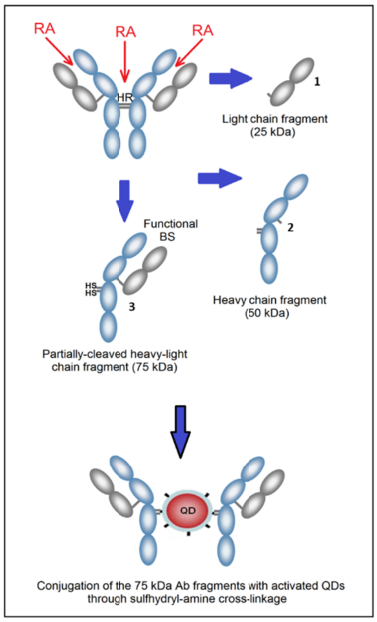

Fig. 2. An optimized protocol for conjugation of antibodies (Abs) and nanoparticles (quantum dots, QDs). In the conjugates obtained using this protocol, the Ab binding sites (BSs) remain intact, which ensures the highest affinity of the conjugate. Ab reduction is made with reducing agents (RAs). The conjugation of QDs with Ab fragments (3) yields nanoprobes with improved functionality of ligand-specific recognition and binding. Adapted from Mahmoud et al. (2011).

3. Results and discussion

Solid planar (Sukhanova and Nabiev (2008)) and suspension (also referred to as particle-based) arrays (Sukhanova et al. (2007); Joumaa et al. (2006); Stsiapura et al. (2004); Brazhnik et al. (2015) are the two common approaches to multiplexed detection, imaging and quantifying of entities available in crude biological probes. The planar array involves depositing two-dimensional grids of probe molecules (Abs, oligonucleotides, candidate drugs, etc.) onto flat solid supports, with each array location acting as a probe for a known target molecule. The suspension arrays technology is based on the use of suspension of optically encoded polymeric microbeads. The microbeads have significant advantages over planar arrays in terms of the way they are produced and used. Indeed, probe molecules can be conjugated to millions of microbeads in separate batches using a variety of proven chemistries under the conditions that are optimal for each probe. Each set of microbeads contains an individual optical code (color combination) that allows the identification of the probe molecules attached to their surface. The probes being identified, the target molecules bound to them can, in turn, be identified in the same way as the molecules bound to 2D planar arrays. Although an encoded bead can move in any direction, the type of code used in multiplexed assays is fixed and remains unchanged during the assay. Hence, whereas planar arrays rely strictly on positional encoding, suspension arrays employ a number of encoding schemes. In contrast to planar arrays, microbead assays are flexible and allow for an easy change of panel by adding or removing microbeads with some or other probes. The microbead array provides the advantages of fast binding kinetics and facilitated separation step. When many microbeads are used for each target molecule in the same assay, rigorous statistical scrutiny of the data is possible, which ensures high-quality results.

Compared with individual dye labels, dye-tagged fluorescent beads (Fig. 3) have a significantly brighter emission due to the high local concentration of fluorophores within the microbeads Additionally, the bead matrix allows protection of the fluorophores from photobleaching, quenching agents, and changes in pH and ionic strength, thus stabilizing the fluorescence emission signal. As the fluorophores are inside the beads, surface groups on the beads are available for conjugation with various capture molecules, such as Abs, and the conjugation step does not normally affect their fluorescent properties. However, the use of microbeads tagged with organic dyes suffers from limitations inherent to these fluorophores. Organic dyes are sensitive to photobleaching, and they are often not bright enough to allow quantitative signal detection in the presence of background fluorescence. Moreover, their fluorescence spectra are not symmetric, and each fluorophore is characterized by its specific optimal wavelength of excitation, which limits their

multiplexing potential. The use of QDs for tagging microbeads permits overcoming these problems. In addition to their extremely high brightness and photostability, QDs of different colors can be excited at the same excitation wavelength and have ideally symmetric fluorescent spectra, thus providing unique possibilities for multiplexing with microbeads, without overlapping of signals from different labels (Sukhanova et al. (2007); Brazhnik et al. (2015)).

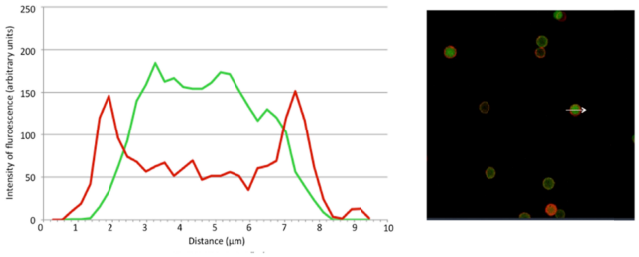

Fig. 3. QD-encoded beads prepared by incubation of nanoparticles of different colors with porous silicon microbeads. It can be seen that the larger QDs with red fluorescence are located on the surface of the beads, whereas the smaller QDs, with fluorescence in the green region of the optical spectrum, can penetrate into the microbeads. The image on the left shows confocal microscopy data confirming this conclusion. Here, the red line shows the distribution of fluorescence intensity of the larger QDs (with red fluorescence) relative to the distance from the surface of the bead, and the green line shows this distribution for the smaller QDs with fluorescence in the green region of the spectrum.

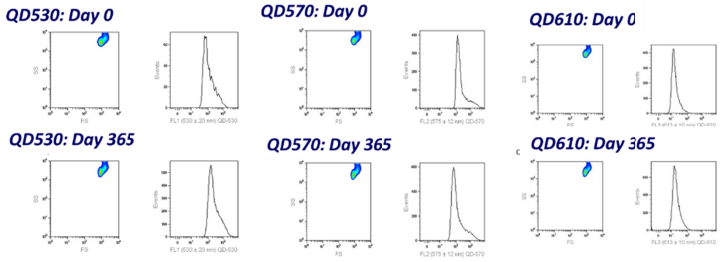

Fig. 4. Stability of QD-encoded beads during their storage for 1 year in an aqueous solution at +4°C. No detectable variation of PL intensity of the beads is detected using flow cytometry.

It is worth mentioning that the application of the B4-principle enabled development of the diagnostic microbeads with unique parameters. Optical properties of such beads were proved to be completely stable at their storage for 1 year without any detectable variation of fluorescence intensity of beads as detected with flow cytometry (Fig. 4).

4. Conclusion

The B4 principle of development of water-soluble nanoprobes paves the way to engineering of a new generation of diagnostic nanophotonic labels with unique optical properties based on highly oriented conjugates of capture molecules with nanoparticles or with optically encoded multiplexed microbeads. The special attention should be paid to nanotoxicity issues and processes of interaction of nanoparticles with the serum proteins (Shemetov et al. (2012)), like serum albumins (Fleury et al. (1997a); Fleury et al. (1997b)), upon penetration of the nanoparticles in the blood and tissues. These issues should be addressed in full if the applications of nanoparticles to invasive diagnostics will be considered in a future.

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